

## Original Research

### Nanolipoparticles-mediated *MDR1* siRNA delivery: preparation, characterization and cellular uptake

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## Abstract

**Objective(s):** Lipid-based nanoparticles (NLP) are PEGylated carriers composed of lipids and encapsulated nucleic acids with a diameter less than 100 nm. The presence of PEG in the NLP formulation improves the particle pharmacokinetic behavior. The purpose of this study was to prepare and characterize NLPs containing MDR1 siRNA and evaluate their cytotoxicity and cellular uptake. MDR1 siRNA could be used in multidrug resistance reversal in cancer therapy.

**Materials and Methods:** siRNAs were encapsulated into NLPs consisted of mPEG-DSPE/DOTAP/DOPE (10:50:40 molar ratio) by the detergent dialysis method. The particle diameters of NLPs and their surface charge were measured using dynamic light scattering. siRNA encapsulation efficiency was determined by an indirect method via filtration and free siRNA concentration determination. NLPs cytotoxicity was investigated by MTT assay. The ability of NLPs for siRNA delivery checked in two human cell lines (MCF-7/ADR and EPP85-181/RDB) by fluorescence microscopy and compared with oligofectamine.

**Results:** NLPs containing MDR1 siRNA were prepared with the stable size of 80-90 nm and the zeta potential near to neutral. The siRNA encapsulation efficacy was more than 80%. These properties are suitable for in vivo siRNA delivery. NLPs cytotoxicity studies demonstrated they were non-toxic at the doses used. NLPs improved siRNA localization in both cell lines.

**Conclusion:** NLPs containing MDR1 siRNA can be a good candidate for in vivo siRNA delivery studies.

**Keywords:** Nanolipoparticles; siRNA delivery; PEG-shielded; Gene therapy; Liposome.

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### Introduction

The use of the RNA interference (RNAi) through the exploiting of small interfering RNA (siRNA), a 21–25 nucleotide (nt) double-stranded RNA molecule, is a powerful tool for post-transcriptional gene silencing. RNAi is being exploited as a promising approach for efficient gene silencing for therapeutic applications like MDR1 mediated multidrug resistance (MDR) reversal in cancer therapy. *MDR1* is the gene that encodes P-glycoprotein (P-gp). The over expression of P-gp is a major mechanism of drug resistance in several cancer types such as breast and pancreatic cancers (1).

But actually the clinical siRNA application is barricaded by some major Problems. For instance, rapid enzymatic degradation of siRNA results in a short blood circulation time and its negative charge leads to poor cellular uptake. So, therapeutic application of siRNA requires developing efficient carriers.

Among the vectors developed for gene delivery, lipid based delivery systems have proved to be one of the most effective carriers (2). Cationic liposomes containing nucleic acids also known as Lipoplexes, are the most commonly used systems for nucleic acids delivery (3). Traditional lipoplexes such as Lipofectamine™ 2000 (4) and Oligofectamine™ (5) are frequently used, commercially available products for both DNA and siRNA delivery.

Despite the widespread use of lipoplexes for *in vitro* experiments, these agents are not efficient gene delivery systems *in vivo*. Excessive size and positive charge result in strong interactions with serum proteins and sequestering within the reticuloendothelial system (RES) (half-life < 5 min) (2, 6). The liposomal nanoparticles with the general name of NP (2) or lipid nanoparticles (LNs) (7) are the other group of liposomes for nucleic acid delivery. NP with small particle size (< 100 nm) and encapsulated nucleic acids in a polyethylene glycol (PEG) -shielded

bilayer shell are different from traditional lipoplexes. Wheeler *et al.* (8) exploited a detergent dialysis method for plasmid DNA encapsulation in nanoparticles named ‘stabilized plasmid-lipid particles (SPLP). In their study, liposome aggregation was prevented during detergent dialysis using 10 mol % of PEG–lipid in the formulation. SPLP with small sizes (<100 nm) and neutral charges have better stability *in vivo* (half-life: 1-10 h) (9) and are suitable for *in vitro* and *in vivo* DNA delivery(10). This procedure was then improved by Li *et al.* (11) who developed a low concentration detergent dialysis method to prepare DNA containing nanolipoparticles (NLPs).

They reduced octyl glycoside (OG) concentration from 200 mM in SPLP preparation procedure to 28 mM (near to critical micelle concentration (CMC)) in NLPs. They increased DNA encapsulation ratio from 40-50% in SPLP to 80-100% in NLPs and improved the transfection activity.

As NLPs have shown high efficiency for gene delivery in *in vitro* studies (2, 11) and have a good potential for *in vivo* applications, they can be a promising candidate for more siRNA delivery studies in different phenomenon like multidrug resistance reversal in cancer. Based on these facts, in current study, NLPs containing siRNA were prepared with the low concentration detergent dialysis method. Physical characteristics, siRNA encapsulation efficiency and stability of NLPs were studied. Cytotoxicity of NLPs was checked with MTT assay. After characterization, the ability of NLPs for siRNA cellular uptake facilitating was investigated by fluorescence microscopy *in vitro*.

### Materials and Methods

#### Materials

1,2-Dioleoyl-3-Trimethylammonium-propane (DOTAP), 1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamine (DOPE), 1,2-distearoyl-sn-glycero-3-phosphoethan

olamine-N-[methoxy(polyethelene glycol)-2000] (mPEG 2000-DSPE) were from Avanti Polar Lipids (Alabaster, AL, USA). Octyl  $\beta$ -D-glucopyranoside (OG) and Tris (hydroxymethyl)aminomethane and Diethyl pyrocarbonate (DEPC) were purchased from Invitrogen™ (USA). Oligofectamine™ (OFA) Transfection Reagent was purchased from Invitrogen™ (USA). Deionised water was used in all experiments and then treated with DEPC for siRNA containing samples. All other chemicals used were of molecular grade.

### siRNA

Two following siRNAs were used for liposome characterization studies: The anti-*MDR1*-specific siRNA duplex (siM) homologous to nt 88-108 (sense strand 5'-GAA ACC AAC UGU CAG UGU AdTdT-3') was previously designed against the *MDR1*-encoding mRNA consensus sequence (GenBank accession number no. NM\_000927)(5) and a biologically active siRNA 5'-CGU ACG CGG AAU ACU UCG AdTdT-3' directed against the non-mammalian luciferase (GL-2) encoding mRNA (siC). In addition, FITC labeled anti-*MDR1*-specific siRNA (siF) (The same sequence as siM) was used for siRNA localization studies. All siRNAs were commercially obtained from Dharmacon (Lafayette, CO, USA) in annealed form.

### Preparation of NLPs containing siRNA

siRNA was encapsulated into NLPs containing mPEG-DSPE/DOTAP/DOPE (10:50:40) by the detergent dialysis method (11) with some modifications. The charge ratio of siRNA and DOTAP was 1:2. Briefly, mPEG-DSPE/DOTAP/DOPE (2.5  $\mu$ mol total lipid) were mixed at a molar ratio 10/50/40 in chloroform (35  $\mu$ L mPEG-DSPE (20 mg/ml) / 34.92  $\mu$ L DOTAP (25 mg/ml) / 37.2  $\mu$ L DOPE (20 mg/ml)), dried by rotary evaporation, placed under a high vacuum for 2 h. Then, the dried lipid film was hydrated in 1.47

ml of 28 mM OG in 5 mM Tris buffer (pH 8.0) for 30 min. 14881<sup>pmol</sup> siRNA was separately solubilized in 1.47 ml of 28 mM OG in 5 mM Tris buffer (pH 8.0). The latter solution was gently added into the lipid solution and vortexed for 30 s. Following 10 min incubation at room temperature for spontaneous particle formation, the mixture was transferred to a Slide-A-Lyzer™ dialysis cassette (MWCO 10 K; Pierce, Rockford, IL, USA) and dialyzed against 1 L of 5 mM Tris buffer (pH 8.0) for 2 days at 4°C. For the *in vitro* study, the buffer was changed to 150 mM NaCl, 5 mM Tris buffer (pH 8.0) by dialysis.

### siRNA encapsulation efficiency assay

siRNA encapsulation efficiency was determined in triplicate by filtration via Amicon Ultra-4 centrifugal filter device, 100 KD. First total formulation filtrated and free siRNA concentration determined in the filtrate by measuring absorption at 260 nm using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Next total formulation lysed by 2% OG (12) and total siRNA concentration was determined as described. The siRNA encapsulation efficiency was calculated using Equation 1.

Equation 1:

$$\text{Encapsulation (\%)} = (1 - \text{Free siRNA} / \text{Total siRNA}) \times 100.$$

### Particle size and zeta-potential measurements

The particle diameters of empty and loaded NLPs were measured in triplicate using dynamic light scattering (ZetaSizer Nano-ZS; Malvern Instruments Ltd., Worcestershire, United Kingdom). The zeta-potential ( $\zeta$ ) was measured with the same instrument using the zeta potential mode as the average of 20 measurements by diluting the particles into a low salt buffer (1 mM NaCl and 1mM HEPES at pH 7.5) (11, 13).

### **Cell lines and cell culture**

MCF-7/ADR and EPP85-181/RDB, established classical multidrug-resistant derivatives of human breast and pancreatic carcinoma cell lines respectively, (from Lage's laboratory stocks) were grown in Leibovitz L-15 medium (Biowhittaker, Walkersville, MD) supplemented by 10% fetal calf serum (FCS) (GIBCO/BRL, Grand Island, NY), 1 mM L-glutamine, 6.25 mg/l fetuin, 80 IE/l insulin, 2.5 mg/ml transferrin, 0.5 g/l glucose, 1.1 g/l NaHCO<sub>3</sub>, 1% minimal essential vitamins and 2x10<sup>4</sup> kIE/l trasylol in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C as described previously (14).

In order to ensure maintenance of drug-resistant phenotype, MCF-7/ADR and EPP85-181/RDB mediums were respectively supplemented with 0.05 µg/ml doxorubicin or 2.5 µg/ml daunorubicin (Farmitalia Carlo Erba, Freiburg, Germany) and were regularly replaced twice a week.

### **NLPs cytotoxicity analysis**

MCF-7/ADR Cells were seeded in clear 96-well plates at a density of 1 x 10<sup>4</sup> cells/well. After 24 h, the cells were transfected at 30–40% confluence. Transfection was performed in OPTI-MEM1 (Invitrogen™, USA) without serum or antibiotics(5). NLPs containing control siRNA (NLP siC) and NLPs containing *MDR1* siRNA (NLP siM) were added at final concentrations of 25, 50, 100 or 200 nM siRNA. Final concentrations of siRNA were calculated based on siRNA concentration in the formulation (5 µM). Empty NLP was added at lipid concentrations equivalent to loaded NLPs. Nontransfected cells were considered as negative controls.

Cells were incubated at 37°C for 3 days in a 5% CO<sub>2</sub> atmosphere. Cell numbers were evaluated using the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (15). Assays were carried out in triplicate and were repeated three times.

### **Translocation studies**

MCF-7/ADR and EPP85-181/RDB Cells were seeded on cover slips (Sigma, USA) in 12 well plates at a density of 1.5 x 10<sup>5</sup> cells per well. After 24 h, cells were transfected with NLPs and OFA containing 25, 50 or 100 nM FITC-labeled siRNA (siF) as described. Untreated cells, cells incubated with naked siF 100 nM and empty liposomes were used as controls. In the case of NLP siF 100 nM, transfection was performed in serum free and complete media for comparison. After 4 h, cells were directly fixed in 4% formaldehyde in PBS for 10 min for fluorescence microscopy, using a Keyence BZ-8100E with excitation-mode for green dye (FITC) (16).

## **Results and Discussion**

### **Liposome characterization**

The biophysical properties of these particles including the z-average particle diameter, the extent of siRNA encapsulation and the zeta potential were determined as described. The mean particle size for empty NLPs, NLPs containing siRNA (NLP siM) were 100.4 ± 36.4, 87.6 ± 6.7 nm (n=3) as calculated by particle size analyzer. To determine the level of positive charge on the particle surface, we measured the zeta-potential by particle size analyzer. The surface charge of NLPs was close to neutral. Zeta potentials for empty NLPs, NLPs containing siRNA were +9.2 ± 4.3, +3.3 ± 10.9 mV respectively. NLPs were stable regarding their size for 10 days at 4°C. The encapsulation efficiency (EE) for NLPs containing siRNA was calculated to be 82.2 ± 4.4%. The results of NLPs containing siRNA characterization are almost similar to ones achieved for NLPs containing DNA by Li *et al.* (11). They prepared neutral NLPs with diameters ranging from 80–150 nm depending on the lipid composition. The percentages of DNA encapsulation for NLPs were about 80-100%.

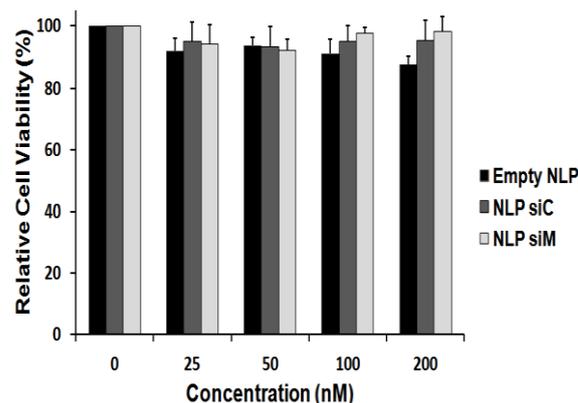
The small size of NLPs as the neutral charge of the surface reduces their interactions with blood components and enables them to escape from RES uptake. So NLPs have the potential for long circulation in animals. In the other hand these NLPs characters help to exploit the EPR effect for passive targeting and make them good candidates for siRNA delivery to tumors(2).

### ***NLPs cytotoxicity***

All formulation including empty NLPs, NLPs containing siM (NLP siM) and NLPs containing siC (NLP siC) at the doses used for transfection were well tolerated by MCF7/ADR cells. There was no significant cytotoxicity ( $P > 0.05$ ) even at the highest dose (200 nM) (Figure 1). These are while Li *et al.* (11) also showed all NLPs tested were well tolerated by monkey kidney fibroblast CV-1 cells at the dose used for transfection and the NLPs containing DNA were better tolerated than traditional lipoplexes. Traditional lipoplexes have high cytotoxicity because of their large size and high positive surface charge. Small size and neutral surface charge of NLPs accompanied with PEG-shielding can reduce blood components interactions and accordingly reduce NLPs cytotoxicity compared with lipoplexes.

### ***Cellular uptake of FITC labeled siRNA***

In order to easily assess the siRNA delivery efficiency of liposomes, MCF-7/ADR (Figure 2) and EPP85-181/RDB (Figure 3) cells were incubated with naked or liposome conjugated FITC labeled siRNA (siF). Fluorescence microscopy studies showed that cellular uptake of siF in both NLPs (Figure 2A and 3A), and oligofectamine<sup>TM</sup> (OFA) (Figure 2B and 3B) transfected cells was dose-dependent. In addition cellular uptake of OFA siF was more than NLP siF in both cell lines. No uptake was observed in control groups (Figure 2C and 3C). We observed no considerable differences between NLP mediated siRNA cellular uptake in serum



**Figure 1.** Cytotoxicity of NLPs containing siC and siM at different concentrations (25, 50, 100 and 200 nM) and empty NLP at lipid concentrations equivalent to loaded liposomes in human multidrug-resistant breast (MCF-7/ADR) cancer cells. The cells were incubated for 72 h with different formulations. Cell number was evaluated using the MTT assay. Means  $\pm$  SD are shown ( $n = 3$ ;  $P > 0.05$ ). Cell viability in untreated cells was taken as control (100%). NLP: Nanolipoparticle; siC: Control siRNA; siM: *MDR1* siRNA

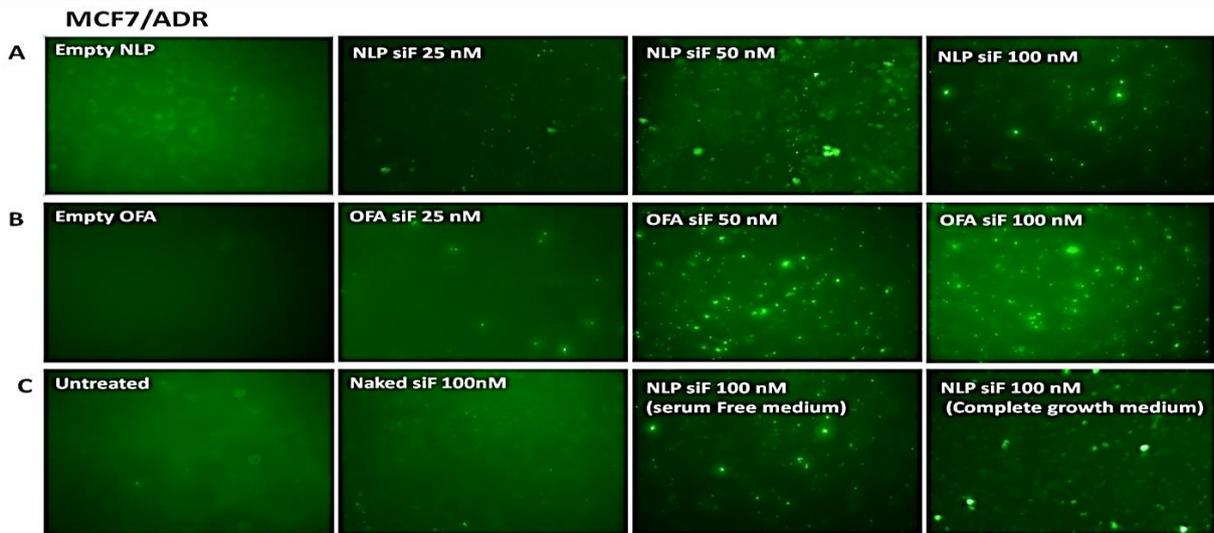
These results can be approved by the results obtained for cell transfection by NLPs containing DNA (11). Li *et al* observed NLPs can deliver DNA to CV-1 cells effectively in the presence of 10% serum. They observed NLPs in comparison to PEI polyplex had lower transfection activity.

The lower transfection of NLPs compared to traditional lipoplexes or polyplexes can be explained with the presence of PEG in the formulation. PEG-lipids reduce the interaction with cell membranes and can decrease the *in vitro* uptake of DNA or siRNA particles into the cells. Nevertheless, PEG-lipid may substantially improve systemic gene transfer by extending the circulation time and reducing the toxicity *in vivo*(2).

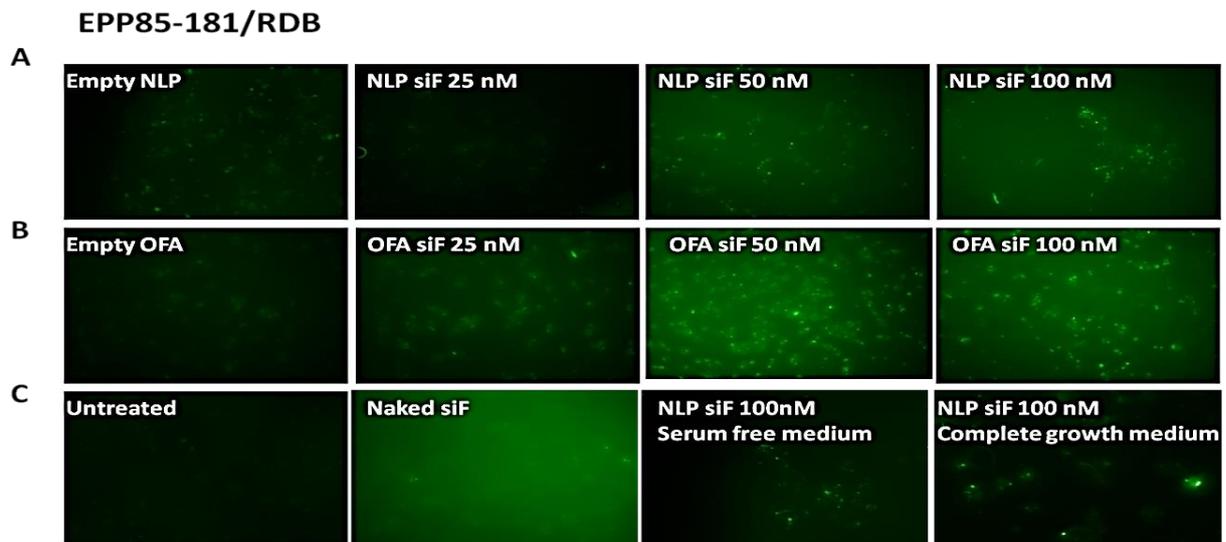
### **Conclusion**

PEGylated nanolipoparticles (NLPs) containing *MDR1* siRNA were prepared with a simple method, based on low concentration detergent dialysis. NLPs with the small size of 80-90 nm and the surface charge close to neutral kept their stability for 10 days.

## Nanolipoparticles-mediated siRNA delivery



**Figure 2.** Translocation of the siF into human multidrug-resistant breast (MCF-7/ADR) cancer cells using different NLP or OFA formulations. The cells seeded on coverslips and were incubated with different formulation at 37 °C without FBS for 4 h. Then cells fixed in 4% formaldehyde and slides were prepared for fluorescence microscopy evaluation. Images were photographed by Keyence BZ-8100E fluorescence microscope. (A) Empty NLP, NLP siF in 25, 50 or 100 nM concentrations. (B) Empty OFA, OFA siF in 25, 50 or 100 nM concentrations. (C) Untreated cells, naked siF100 nM treated cells used as controls. Serum effects on NLP mediated translocation was evaluated by NLP siF 100 nM tranfection in 10% FBS medium. NLP: Nanolipoparticle; OFA: Oligofectamine; siF: FITC labeled siRNA.



**Figure 3.** Translocation of the siF into human multidrug-resistant pancreatic (EPP85-181/RDB) cancer cells using different NLP or OFA formulations. The cells seeded on coverslips and were incubated with different formulation at 37 °C without FBS for 4 h. Then cells fixed in 4% formaldehyde and slides were prepared for fluorescence microscopy evaluation. Images were photographed by Keyence BZ-8100E fluorescence microscope. (A) Empty NLP, NLP siF in 25, 50 or 100 nM concentrations. (B) Empty OFA, OFA siF in 25, 50 or 100 nM concentrations. (C) Untreated cells, naked siF100 nM treated cells used as controls. Serum effects on NLP mediated translocation was evaluated by NLP siF 100 nM tranfection in 10% FBS medium. NLP: Nanolipoparticle; OFA: Oligofectamine; siF: FITC labeled siRNA.

The efficacy of siRNA encapsulating in NLPs was more than 80%. The NLPs formulations didn't have any negative effect on cell viability, as tested by MTT assay. These characters make them a good candidate for in vivo studies. The results of fluorescent imaging by microscopic studies showed the cellular uptake of siRNA in resistant cancer cells is suitable and this formulation merits further in vivo studies for multidrug resistance reversal in cancer therapy.

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